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What's bad in cancer is good in the embryo: Importance of EMT in neural crest development

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Abstract

Although the epithelial to mesenchymal transition (EMT) is famous for its role in cancer metastasis, it also is a normal developmental event in which epithelial cells are converted into migratory mesenchymal cells. A prime example of EMT during development occurs when neural crest (NC) cells emigrate from the neural tube thus providing an excellent model to study the principles of EMT in a nonmalignant environment. NC cells start life as neuroepithelial cells intermixed with precursors of the central nervous system. After EMT, they delaminate and begin migrating, often to distant sites in the embryo. While proliferating and maintaining multipotency and cell survival the transitioning neural crest cells lose apicobasal polarity and the basement membrane is broken down. This review discusses how these events are coordinated and regulated, by series of events involving signaling factors, gene regulatory interactions, as well as epigenetic and post-transcriptional modifications. Even though the series of events involved in NC EMT are well known, the sequence in which these steps take place remains a subject of debate, raising the intriguing possibility that, rather than being a single event, neural crest EMT may involve multiple parallel mechanisms.

Keywords

neural crest; EMT; apicobasal polarity; dorsal neural tube; Snail

1. Introduction

During development, most growing cell populations undergo several rounds of transition from their epithelial phenotype into a mesenchymal fate (Epithelial to Mesenchymal Transition, EMT) and/or vice versa (Mesenchymal to Epithelial Transition, MET). Epithelial cells are tightly bound to each other with tight junctions, adherens junctions, desmosomes and gap junctions, which together form coherent apicobasally polarized cell layers. These organized epithelial structures are surrounded with an underlying basement membrane, which physically separates them from other tissues. In contrast, unpolarized mesenchymal cells are not attached by intercellular adhesion complexes. This enables them to respond quickly to environmental cues and to move as individuals throughout the extracellular matrix. Thus, migratory cells display less polarity than epithelial cells, though often communicate with neighboring cells in order to coordinate their movements. EMT enables

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the construction of various complicated organs with lumens, lobes and separating barriers. However, primary EMT only takes place twice in the embryo, during gastrulation and neural crest formation [1, 2].

The neural crest (NC) provides an excellent model to study the principals of EMT in a nonmalignant environment. Neural crest precursors initially reside within the forming central nervous system but subsequently undergo EMT to migrate to distant locations in the embryo and then differentiate into diverse derivatives. Because neural crest EMT occurs in the early embryo, most knowledge is acquired by using model vertebrate organisms that develop externally such as the frog, chicken and fish.

Upon differentiation, neural crest cells form many derivatives that are prone to malignant transformation, including melanocytes that can form melanomas and glial cells that can form Schwannomas and gliomas. Cancer growth often relies upon reactivation of several developmental cellular programs such as maintenance of the self-renewal and proliferation capacity. In order for the cancer cells to leave the original tumor, EMT machinery is activated, epithelial adhesion is broken down and the metastatic cells escape through the ruptured basement membrane into the circulation. Conversely, MET is needed for the reverse actions at the target site. Since the recognition that EMT is one of the hallmarks of cancer, interest in cancer EMT research has expanded rapidly. Reactivated EMT is also the cause of organ fibrosis, which leads to the loss of functionality of the epithelial structures (e.g. renal tubules) and thus the whole organ [3–5].

2. The neural crest

The neural crest (NC) is a transient embryonic cell population that forms in the dorsal parts of the newly formed neural tube. The NC cells subsequently undergo EMT, and begin to migrate through the mesenchyme of the developing embryo to give rise to the peripheral nervous system, cranial bone and cartilage, melanocytes and parts of the adrenal medulla. The gene regulatory network (GRN) that orchestrates NC induction and specification is evolutionarily conserved in vertebrates [6–10].

Briefly, induction of NC starts during the gastrula stage [11, 12]. A combination of BMP, Wnt and FGF signaling is important for setting up the neural plate border territory. At these stages, BMP (BMP 4 and 7 in the chicken) secreted by the epidermal ectoderm promotes dorsalization and antagonizes Shh from the ventral midline [13, 14]. Wnt-signals, also secreted by the epidermal ectoderm (Wnt8 in fish, frog and lamprey, Wnt6 in chicken) are also critical for NC formation [6, 15–17], as is FGF signaling secreted from the paraxial mesoderm [18].

These extracellular signals regulate expression of a group of essential transcription factors called the neural plate border specifier genes (or dorsal patterning genes). In the frog for example, *Msx1* induces expression of *Pax3* and *Zic1* [19], which together are sufficient to induce NC specifier genes [19–21]. In the chick *Pax7*, which is expressed in the neural plate border beginning at gastrula stages, is required for specification of NC fate and seems to have a more critical role than *Pax3* [11]. In some species like mouse, *Pax3* and *Pax7* may exhibit functional redundancy but have later defects in neural crest derivatives [22, 23]. The neural plate border specifiers in turn activate the transcription of the NC specification genes (e.g. *Snail1/2*, *SOXE*, *FoxD3*, *AP-2* and *c-Myc*) in the dorsal neural folds [9, 20, 24, 25].

These neural crest specifier genes are thought to switch on the EMT program in a subset of cells in the dorsal neural tube. These detach from the neural epithelium and become pluripotent migratory mesenchymal cells. There are likely to be many additional regulators in the NC GRN, that together orchestrate a complex series of cellular events that allow

neural crest cells to emigrate from the neural tube. Various mechanisms, ranging from epigenetics to regulatory RNAs and posttranslational protein modifications, are likely to work coordinately to fine tune the activity of key NC players to contribute to the spatiotemporal control of EMT. Finally, the migrating NC cells assume one of many possible fates either during their migration or upon arriving at their final destination and differentiate into their target tissue [9, 26].

3. The Epithelial to Mesenchymal Transition

Traditionally, EMT is thought to consist of a breakdown of the epithelial adhesion components, loss of apicobasal polarity and rupture of the basement membrane, which then leads to mesenchymal motility. However, these functions overlap and the cell also needs to maintain multipotency, survival and overall adhesive integrity of the source tissue [27]. In this review we focus on what is known about the regulation of EMT in the NC. The “basic rules” apply to all EMT from gastrulation to carcinoma [3], although as discussed below, different individual molecular players fulfill the same tasks in different tissues, species and even at different axial levels, such as the cranial versus trunk region of the neural crest even within the same organism.

3.1 Polarity, adhesive junctions and basement membrane during NC EMT

3.1.1 Adherens junctions and cadherin expression during EMT—Adherens junctions (AJ) are cell-cell adhesion complexes that are comprised of classical calcium dependent cadherins and catenins. These include type I cadherins (E-, N, P- and R-cadherin) and type II cadherins (cadherin 5 and beyond), the extracellular domains of which form a homophilic bond with the cadherins on adjacent cells. The intracellular domain forms a complex with the catenin proteins in order to strengthen the adhesion by connecting with the actin cytoskeleton (the cadherin binds p120- and β -catenin and β -catenin binds α -catenin) or microtubule cytoskeleton (β -catenin, p120catenin) or both [28]. A repertoire of actin-associated proteins including vinculin, α -actinin, formins, ZO1 (also associated with tight junctions), and EPLIN are involved in the binding of α -catenin to the actin filaments [28, 29].

The homophilic interactions of cadherins serve as adhesive sorting factors that mediate morphogenesis of different cell types. During the formation of the neural tube, which is a pseudostratified epithelium, the expression of E-cadherin is gradually downregulated and switched to N-cadherin (N-cad) in the neural progenitors of the neural plate. The non-neural ectoderm continues to express E-cadherin, which thus keeps these two structures separated. N-cad expression is most prominent in the apicolateral adherens junctions of the neural tube where it presumably maintains the apicobasal polarity as well as the adhesive integrity of the neural tube. [30–32]. The neuroepithelial cells also express another classical cadherin, Cad6B, that is first transcribed in the whole neural plate (although most intensively dorsally) and later, prior to neural tube closure, restricted only to the cells in the dorsal neural folds/neural tube [14, 33–35]. Both *N-cad* and *Cad6B* transcripts are downregulated from the dorsal neural tube prior to emigration of neural crest cells whereas *Cad7/11* expression comes on in the migratory crest [33, 34, 36, 37]. In addition to the localization of Cad6B at the apical membrane cell junction complexes, it (as well as β -catenin) is also expressed in a more nonpolarized fashion ubiquitously throughout the cytoplasm in the dorsal neural tube [35].

N-cadherin during EMT: The hallmark of the EMT program during gastrulation as well as in carcinomas is the direct transcriptional repression of E-cadherin by Snail, which leads to a loss of adherens junctions and cell polarity [4, 32]. In the avian NC, N-cad is lost from the apical membrane junctions prior to emigration [36]. Interestingly, as the full length N-cad

antagonizes EMT, it is proteolytically cleaved from the cell membrane via a BMP triggered, ADAM10 metalloprotease dependent mechanism in the delaminating NC cells. The truncated soluble cytoplasmic form CTF2 localizes in the nucleus and switches from an EMT inhibitor into an EMT promoting transcription factor [38]. N-cad is thought to be downregulated by the transcription factor Foxd3 [39] and overexpression of N-cad inhibits NC delamination, migration and BMP signaling [35, 36, 38]. Even though a breakdown of the apicobasal polarity of the neuroepithelium is required for emigration, the migrating NC cells also display polarity in order to orientate in the correct direction. N-cadherin appears to mediate this reduced contact in migrating NC cells, and is involved in the regulation of contact inhibition of locomotion (CIL) [1, 40, 41].

Cadherin 6B during EMT: The loss of Cad6B is directly regulated by transcriptional repression of *Snail2* prior to migration of NC in the chick [42]. This was thought to regulate the correct timing of NC emigration and delamination from the neural tube without affecting the expression of the migratory proteins of NC [43]. However, a recent study suggests that the avian Cad6b acts via BMP signaling to trigger de-epithelization of the premigratory NC cells without affecting the actual delamination process or breakdown of the basement membrane [35]. Over-expression of Cad6B caused an ectopic loss of polarity, as demonstrated by disruption of ZO-1 expression that lead to a general lack of epithelial integrity. However, the mesenchymal cells were not able to break through the basement membrane but instead accumulated in the lumen of the NT [35]. Accordingly, shRNA mediated knockdown of *Cad6B* resulted in a lack of depolarization followed by a lack of migration, since the cells had not adopted a mesenchymal phenotype [35]. The seemingly contradictory results from these studies may be due to the different axial differences and timing of performing over-expression and knockdown. For studies of cranial NC [42, 43], translation blocking morpholinos (MO) as well as overexpression constructs were electroporated into the newly formed neural tube at the 2–4 somite stage for *Cad6b* [43] and the 5–7 somite stage for *Snail2* [42]. At this time point the de-epithelization process discovered by Park and Gumbiner (2010) is already strongly ongoing in the cranial premigratory crest (as depolarized Cad6B expression starts in the dorsal neural folds before neural tube closure). Instead, manipulating *Cad6b* and *Snail2* at these later time points likely affects the onset of migration in the cranial NC cells, which indeed operates through Cad6B downregulation. This nicely demonstrates that EMT consists of separately regulated and distinct cellular functions that include NC specification, depolarization, delamination, emigration and migration. Taken the results together, Cad6B appears to be crucial for the loss of polarity in the transition into a mesenchymal cell type and needs to be degraded in order for the proper migration of NC to take place [35, 42]. Further support for this scenario comes from the over-expression phenotype of many different cadherins in the migratory crest [36], all of which block emigration.

A shift from type classical cadherins to more mesenchymal cadherins correlates with the acquisition of cell motility. Premigratory depolarizing NC cells switch N-cad first to Cad6B, which in the migratory cells is again replaced with Cad7 expression in the chick and Cad11 in the frog [26, 33, 44–46].

3.1.2 Rho family of small GTPases control cytoskeleton changes—The members of the Rho family are small G-protein signaling molecules and a subfamily of the Ras superfamily. Rhos participate in various cellular functions including cellular morphology and motility changes by controlling the cytoskeletal rearrangements [47]. In the neurula stage embryo, expression of RhoC is restricted to the notochord. RhoA is ubiquitously expressed in the neural plate and mesoderm but after NT closure it seems to be concentrated in the dorsal lateral part of the NT [14]. *RhoB*, on the other hand was found as one of the NC genes induced by BMP in the neural plate and its expression is restricted to the pre- and

early migratory NC. *RhoB* null mice do not display any evident developmental defects, which may reflect redundancy between the family members [48]. The avian *RhoB* expression is turned on after *Snail2* and does not appear to affect neural crest cell fate specification (i.e. no alteration in expression of NC specific genes *Snail2*, *Sox9*, *Cad6B* or *Foxd3*) or migration. Rather, it has been proposed to play an important role in delamination from the neural tube [14, 49], since neural crest cells failed to emigrate from neural plate explants treated with Rho inhibitors [14]. Although originally shown to be essential for NC delamination and thus the loss of epithelial polarity, a more recent paper indicates that both RhoB and RhoA, through ROCK signaling, act to maintain apicobasal polarity and stabilize N-cadherin in a vinculin based manner at the focal adhesion points. The loss of the membrane bound active form of Rho, on the other hand, was associated with delamination and EMT and cytoplasmic nonpolarised RhoB was detected in mesenchymal cells. Both loss and gain of function studies of RhoB and RhoA supported this finding [49]. Moreover, breakdown of RhoA followed by delamination appears to be essential for EMT during gastrulation [50]. The differences in the studies may be due to the more efficient and less toxic reagents used in the more recent study [49]. RhoA in the migratory post-otic NC influences migration rate and filopodia dynamics [51]. Since the inhibitor used by Liu and Jessell [14] inhibits activity of both RhoB and RhoA, it is possible that instead of affecting delamination, the inhibitor was added at a slightly later timepoint and the noted effects were on migration of already delaminated cells. This coupled with possible differences in the axial levels under study may have contributed to the different observations. Also, as RhoB is expressed in the early migratory NC, similar to RhoA, it may have two distinct roles in epithelial versus mesenchymal cells. RhoA negatively regulates the expression of NC genes (including the cranial crest) *Snail1/2* and *FoxD3* in *Xenopus*. Interestingly the results with another member of the Rho family, Rac that has been associated with non-canonical Wnt signaling in dendrite morphogenesis [52], were the opposite as its overexpression induced the NC markers [53]. Yet another Rho family protein RAP also has a role in the early NC migration in zebrafish, which is controlled by a RAP mediator RADIL [54].

In addition to cell polarity, RhoB mediated signaling is important for the competence of NC to undergo EMT in the chick. Whereas ectopic expression of *Sox9* does not cause EMT, it together with *RhoB* is sufficient to promote EMT. Ectopic RhoB alone, on the other hand, does not induce HNK-1 positive NC cells but results in disturbed morphology and apoptosis. Interestingly, overexpression of *Sox9* and *Snail2* induces EMT, suggesting that there is no epistatic relationship between RhoB and the two other NC signaling inducers, respectively [39]. On the other hand, in the avian hindbrain, an increase in expression was reported after *Snail2* overexpression [55], which might be due to the differences between cranial and trunk NC or to interesting cross-regulation.

3.1.3 Tight junctions and the protein complexes in the polarized apical plasma membrane—Tight junctions, the most apically located cellular junctions also contribute to the apicobasal polarity of the neural epithelium. Adjacent plasma membranes are sealed together by the transmembrane proteins claudins and occludins. Tight junctions are constantly being remodeled via interactions with the interface protein Zona Occludens ZO-1 and are in contact with Rho GTPases by forming complexes with other junctional adaptor proteins like the partitioning defective (PAR), Crumbs and Scribble complexes [56–58]. Tight junctions are prominent in the neural plate but disappear soon after closure of the NT. Loss of tight junctions is associated with an increase in N-cadherin expression as well as maintenance of ZO-1 in the apical membranes [57]. Adherens junctions also maintain polarity by communication with the conserved intracellular apical protein complexes which, in *Drosophila* neuroectoderm, are controlled by Cdc42 and PAR-proteins [59]. The Crumbs complex protein Pals is also expressed in the apical junctions in the avian NT [35]. After loss of tight junctions, maintenance of apical neuroepithelium relies also on tiny cholesterol

based prominin-1 containing microdomains (called lipid rafts) of apical membrane protrusions, which also play a major role in the asymmetric division of the neuroepithelial cells [60–62]. Finally, gap junctions replace tight junctions during neural tube closure in the frog [63]. Mouse premigratory NC cells start to express the essential gap junction protein connexin 43, which is particularly important in the cardiac NC [64].

3.1.4 The basement membrane and extracellular matrix—The basement membrane, composed of type IV collagen fibers and different glycoproteins (e.g. laminins, dystroglycans, proteoglycans) separates the neural epithelium from the mesenchyme. The extracellular matrix (ECM) proteins, such as the ADAM proteins and matrix metalloproteinases (MMP), via signaling through integrin and cadherin receptors control the basement membrane and induce its breakdown during EMT to allow migration. MMP-2 and ADAM13 positively correlate with NC delamination and migration [26, 65, 66] and several laminins, proteoglycans and integrin dimers, especially in combination with the $\beta 1$ integrin subunit, support the migratory NC phenotype [67–72]. Noelin, a secreted glycoprotein that is expressed in the neural folds at the onset of EMT and is maintained all through NC migration has also been reported to positively correlate with the onset of avian NC emigration [73].

3.2 Inductive signals of NC EMT

3.2.1 Notch, BMP, FGF and Wnt signaling—The NC cells are specified at the neural plate border and will obtain a unique cell fate as compared to the prospective cells of the non-neural ectoderm as well as the neural cells, respectively. Notch signaling promotes NC fate in the neural plate in chicken, fish and frog. Induced by the Delta1 ligand expressed by the neighboring endodermal ectoderm cells Notch signaling is needed to induce BMP4 expression in the prospective NC and also to inhibit *Snail2* from being expressed too early in the chicken [74–76]. BMP (BMP4 in chicken, BMP2 in mouse), a member of the TGF β family, secreted in the dorsal NT (as opposed to the BMPs secreted by the epidermal ectoderm needed for NC specification at the neural plate border) induces EMT in the premigratory NC cells and turns on expression of essential NC premigratory genes including, *RhoB*, *Cad6B* and *Snail2* [14, 38, 77] although the results on *Snail2* activation are controversial [78]. Zebrafish mutants of the BMP2 signal transducing pathway display reduction of the NC formation [79] and Smad1 directly binds the chicken *Snail2* promoter (which when expressed in the mouse mimics the expression pattern of mouse *Snail*) and activates EMT [80]. The rostral BMP signal competes with caudally expressed Noggin to establish a rostrocaudal gradient of increasing levels of active BMP in the dorsal NT. In the avian trunk, The BMP/Noggin gradient is controlled by the developing somites, which thus regulate the timing of NC EMT as illustrated in figure 1 [78, 81–83]. It is important to note that BMP4 has also been shown to induce apoptosis (via *Msx2*) in the hindbrain NC [84] and high levels of *Snail*/*Snail2* expression in both mouse and chick hindbrains are sufficient to overcome the BMP induced cell death in the emigrating cells, in which the level of BMP4 expression is again low [85].

It was recently shown that FGF4/8 secreted by the somites, which signals NC induction at the neural plate border, maintains Noggin expression in the caudal neural tube (as discussed in 3.2.2). Ectopic FGF inhibited expression of NCC specifier genes *Snail2* and *Sox10*. Like the BMP4/Noggin gradient, FGF is also expressed in a rostrocaudal gradient and inhibited by retinoic acid (RA) signaling (Figures. 1A,B). However, RA is not able to directly downregulate Noggin but instead functions through activation of Wnt1, which in turn promotes BMP4 secretion and triggers EMT (Fig. 1C). RA was needed for the onset of NC emigration but not for the expression of NC specification genes such as *Snail2* or *FoxD3* [86]. Also earlier studies have suggested that BMP induces Wnt1 signalling in the avian

trunk NC, which leads to NC delamination through activation of a CyclinD driven transition of the cell cycle from G1 arrest to DNA synthesis (S) phase and Wnt signaling was essential for the avian expression of *Cad6B*, *Msx1* and *Pax3* [78]. In line with this, the presence of Wnt1 is required for the transcriptional activation of *Snail2* by *Pax3* and *Zic1* in the *Xenopus* neural folds in a pathway at least partially mediated by RhoV [19, 87]. Moreover, Wnts and BMPs upregulate transcription of NC genes in explanted chick neural plates [88]. Cv-2 and Cad6B also promote BMP activity in the avian NT [35, 89]. Cad6B links the role of BMP particularly with the onset of de-epithelization of the polar premigratory NC cells and might activate BMP signaling at the receptor level in the absence of ligand [35].

BMP4, which is a member of the TGF β family, may function in combination with the canonical Wnt-signaling together with N-cadherin. N-cadherin inhibits NC delamination physically by maintaining adhesion junctions but also by storing β -catenin in the adhesive membrane complex [38]. As mentioned earlier, BMP4 triggers cleavage of N-cadherin via the metalloprotease ADAM10. The cleaved intracellular domain, CTF2, activates transcription of *Cyclin D* and β -catenin, which further enhance de-epithelization by direct transcriptional activation of other EMT genes like *Snail2* in the frog [38, 90]. This is similar to events during gastrulation, renal fibrosis and some metastatic cancers, where TGF β together with Wnts are the main inducers of EMT [4, 91–94]. For example, during gastrulation, Wnt signaling is needed for the competence of the ectoderm to undergo EMT, which is then induced by the TGF β -superfamily members Nodal and Vg1 [95, 96]. FGF signaling also controls EMT and the maintenance of the mesodermal phenotype by positively regulating *Snail*, *Brachyury* and *Tbx6* expression during gastrulation [97].

3.2.2 Regulation of genes at the neural plate border before EMT—MAPK-signalling pathway, downstream of FGF8 signalling, is needed for neural crest specification. In the frog neural plate, FGF8 induces *Msx1* expression, which in turn, in the presence of Wnt signaling, drives expression of *Pax3* and *Zic1*. In turn, *Pax7* is crucial for the NC specification in the chick, see Figure 1B [11, 15, 19, 86]. However, once the premigratory NC cells are specified, FGF8 needs to be downregulated via RA to allow activation of *Snail2* and other NC genes e.g. *Foxd3*, *Sox9* and *Sox10* and *Sox5* (Fig 1A,C). In line with this, the RA synthesizing gene *Raldh2* spatiotemporally coincides with the increasing avian *Snail2* mRNA [86]. It was recently proposed that the anterior Hox-genes also mediate NC specification and expression of *Msx1/2* in a Notch and BMP dependent manner in the cranial NC [98]. In the frog, BMP and Wnt signaling also induce the expression of the transcription factor AP-2, yet another NC specifier that induces expression of *Sox9* and *Snail2* and marks the NC forming territory at the neural plate [99].

3.3 EMT inducing transcription factors in the neural crest

All of the NC specifier genes *Snail/Snail2*, *SoxE* and *Foxd3* function separately in order for neural crest EMT to occur in the neural folds/NT. Thus, for completion of EMT, the presumptive NC cells need to acquire competence for the pluripotent NC identity, loose the apicobasal polarity and break down the basement membrane [9, 39]. The induction and maintenance of stem cell properties during EMT is crucial during development and cancer and the realization that EMT generates cells with self-renewing properties holds promises of resolving a major problem in cancer biology [100]. Interestingly, the same transcription factors seem to mediate multiple functions during specification, depolarization and migration of NC [39, 101] as illustrated in Figure 2. Because transcriptional and signaling networks often have positive and negative feedback loops, it is often difficult to parse the original order of the events [8, 10].

3.3.1 Loss of polarity and delamination

Snail family of transcription factors induce loss of epithelial morphology and

migration: Snail/Snail2 has two roles during NC EMT [101]. Snail /Snail2 is the key inducer of EMT marked by the loss of epithelial cell phenotype in NC and the gastrulating embryo [39, 95, 102, 103]. It also has an earlier role during NC specification, which includes maintenance of NC cell survival, which is discussed in 3.3.2. The members of the Snail family of transcription factors all share a similar carboxyl terminal structure that consists of four to six DNA binding zinc finger domains. Snail is the main EMT inducer in mouse premigratory NC whereas only Snail2 (also called Slug) is expressed in the avian dorsal neural folds prior to neural tube closure and remains on during EMT and early migration. The mouse *Snail2* expression comes on only in the migratory NC, which thus expresses both *Snail* and *Snail2* [102–104]. The mouse Snail can induce EMT in the avian crest, which indicates functional equality of the two family members [55]. *Xenopus Snail1* and *Snail2* are both involved in NC specification and EMT. Functional redundancy occurs in frog [105] and mouse NC and thus only the double mutant mice lacking both *Snail2* and *Snail* display a full NC defective phenotype [106]. In zebrafish, *Snail1b* (a *Snail* duplicate, previously called *Snail2* [107]) expression also defines the NC forming cells [103, 108]. Inhibition of avian *Snail2* mRNA (electroporated into the closed NT) blocked emigration from the neuroepithelium, while *Snail2* overexpression increased the delamination of premigratory NC as well as the numbers of HNK-1 positive migratory cells [55, 103]. Overexpression of a dominant negative form Snail2 also inhibits NC emigration in *Xenopus* [101].

Snail proteins repress transcription of target genes by binding to E-boxes in their promoters. For example, in gastrulation, carcinomas and renal fibrosis, Snail induces EMT by directly repressing transcription of *E-cadherin*, [4, 32, 109, 110]. Snail/Snail2 can also repress other cadherins to allow EMT such as Cad16 in renal fibrosis or Cad6B in the NC [42, 111]. Snail2 also directly represses the adherens junction associated neural N-catenin and MO mediated knockdown of *N-catenin* increased the number of emigrating cranial NC cells [112]. Snail also directly represses other polarity genes like *Claudin*, *Occludin* and *Crumbs* in *in vitro* cultured epithelial cells [113, 114]. The Ajuba family of LIM proteins function as corepressors of the Snail family via an interaction with the SNAG domain in the *E-cadherin* promoter in cell lines. The same corepressors are also required for a proper function of Snail2 in *Xenopus* NC [115]. Pax3/7 and Zic activate Snail2 in a Wnt dependent manner [11, 19, 20] and also by direct binding the β -cat/TCF/Lef complex on the *Snail2* enhancer in the frog [90]. The avian *Snail2* promoter is also directly activated through Smad1 signalling downstream of BMP4 signalling [80]. Sox9 directly activates *Snail2* and also autoregulates its own transcription by directly binding to E-Box2. Similarly, in *Xenopus*, Snail1 and Snail2 also are needed to maintain each other [105]. In cancer cells Snail2 is required for *Twist* induced EMT [116].

FoxD3 and Ets1 regulate depolarization and delamination: In all vertebrates, the Forkhead Box family member *FoxD3* expression starts in the premigratory NC of the dorsal neural folds, is shut down soon after the beginning of NC migration and reactivated again in the late migratory crest. Finally, it is downregulated as the NC cells reach their final destinations and differentiate [117–120]. Although knockout models in mice and zebrafish show that lack of Foxd3 does not greatly affect emigration of NC (or expression of *RhoB*, *Snail2* or *Cad6B*), ectopic *FoxD3* affects the transition of epithelial dorsal NT cells into a mesenchymal phenotype. Overexpression of avian *Foxd3* in the trunk resulted in total loss of N-cadherin and an increase of β 1-integrin as well as other mesenchymal proteins such as Laminin and Cad7 [39, 121–123]. The transcription factor Ets1, which is associated with acquisition of cell mobility and invasiveness during development and cancer, is expressed in

the premigratory cranial NC and associated with the breakdown of Laminin and the basement membrane in the avian cranial NC. Ectopic *Ets1* did not affect specification of the NC fate or other steps of EMT and alone could not induce expression of *FoxD3*, *Snail2* or *Sox10* nor promote transition into the mesenchymal fate [124].

Zeb2: The zinc finger E-box binding homeobox transcriptional repressor family member Zeb2/Sip1 is a known EMT inducer in cancer cells that is activated by Tgf β /Smad signaling. It can directly repress transcription of E-cadherin as well as other genes involved in epithelial cell junctions and can also function indirectly by repressing miRNAs [125–127]. *Xenopus* Zeb2 expression starts in the developing neural plate and continues in the premigratory and migratory NC cells [128]. In mouse and frog Zeb2 is thus involved in the specification of the neuroepithelium. It regulates expression of the neural progenitor genes *SOX2/3* as well as the neural plate border gene *MSX-1* and Zeb2 activity was regulated by BMP/Smad3 as well as FGF signaling [129, 130]. The lack of Zeb2 dramatically affected specification of the vagal NC that forms the enteric nervous system. Mice lacking Zeb2 also displayed ectopic *E-cadherin* expression throughout the NT and emigration of the cranial NC was inhibited [129, 130].

3.3.2 Neural crest competence, multipotency and cell survival

Sox genes induce NC competence and maintain multipotency: The SoxE genes (*Sox8*, *Sox9* and *Sox10*) are all expressed in the NC and its derivatives and they exhibit both overlapping and unique functions in NC development. Their expression patterns and several functional studies suggest redundancy between the family members [131, 132] and they are crucial for the early specification as well as for the maintenance of multipotency of premigratory and early migratory NC cells as discussed here. However, SoxE genes also display multiple functions during differentiation of NC [10, 132–136].

In *Xenopus* *Sox8* precedes the expression of *Sox9* and *Sox10* and its knockdown mainly induces reduction of the expression of *Sox10* (but not *Sox9* or *Snail2*) and an overall delay in NC induction [137]. In chicken the timing of the onset of *Sox9* expression precedes that of the other premigratory NC markers (*Snail2*, *FoxD3*, *Sox10* and *HNK-1*) and its function is crucial for establishing the competence for NC cells to undergo EMT [39, 136, 138]. Wnt and BMP-regulated NC induction in *Xenopus* and chicken embryos depends on Sox9 transcriptional activator function. Loss of Sox9 in the frog also results in a dramatic loss of NC progenitors particularly affecting the morphology of the craniofacial skeleton [25, 138–140]. Sox9 directly activates *Snail2*, which in turn activates other NC fate inducing genes and also promotes cell survival in the presumptive NC, which without *Sox9* undergo apoptosis and cannot start EMT [39, 138]. In line with this, forced expression of *Sox9* increases the territory of NC producing cells at the expense of cells adopting a neural fate but it does not induce emigration of the avian NC [136]. In the cranial but not vagal or trunk premigratory NC, Sox9 directly activates *Sox10* although *Sox10* levels were reduced in the migratory *Sox9*^{−/−} trunk NC cells, perhaps representing a secondary effect [39, 141]. Sox9 and Sox10 have also been reported to be able to compensate for the loss of each other without directly activating each other since expression of *Sox9* in *Sox10* depleted *Xenopus* embryos rescues NC formation [142]. The knockdown of *Sox9* did not affect the levels of *FoxD3*, suggesting separate regulatory pathways for the key NC specifier genes [39].

Sox10 maintains multipotency of the premigratory and migratory nonmesenchymal (neural, glial and melanocytic) NC progenitors. It seems that *Sox10* is especially crucial for the maintenance of glial potential of the NC stem cells [133, 134, 143] even though a high level of Sox10 expression is later needed for the differentiation of NC derived peripheral neurons [133, 143] and melanocytes [144, 145]. Constant overexpression of avian *Sox10* induces

ectopic NC cell fate despite the absence of dorsal signals in the NT [146]. Knockdown of *Xenopus Sox10* causes a loss of premigratory NC precursors affecting both the proliferation and survival of the cells. Also the expression of other NC specific markers was reduced and the timing of *Sox10* activation was thought to take place in between the activation of *Snail1* and *Snail2* [147]. In addition to direct activation by Sox9, Ets1 and c-Myb also directly bind to the *Sox10* enhancer and thus directly activate *Sox10* [141].

Sox5, a member of the SoxD family, is expressed in the early premigratory NC and its expression seems to be initiated by FoxD3 and Snail2. Ectopic expression of *Sox5* resulted in overproduction of NC in the avian cranial crest [150]. In the mouse melanocytic lineage Sox5 inhibits *Sox10* transcriptional activity by recruiting chromatin modifiers CTbp2 and HDAC1 to the promoter [150, 151].

Foxd3 maintains NC self-renewal and pluripotency: FoxD3 maintains self-renewal and inhibits differentiation in several stem cell types including e.g. embryonic stem cells. In ES cells, inhibition of *Foxd3* induced differentiation and it appears to be downstream of the self-renewal inducing factors Nanog, Oct4, c-Myc and Klf. It seems to directly inhibit transcriptional activity of mesoendodermal differentiation genes like Brachyury and Goosecoid [152–154]. FoxD3 also inhibits the cell cycle inhibitor *p21* [120]. Foxd3 expression is also critical for the survival and self-renewal of NC. Loss of Foxd3 in neural crest targeted Wnt1-cre mice as well as in a zebra fish mutant (mother superior and Sym1, respectively) causes a devastating loss of NC derivatives [122, 155, 156]. FoxD3-mediated maintenance of self-renewal operates through repression of a mesenchymal as well as melanocytic fate (and FoxD3 thus maintains neural potential) and this lineage specification starts in the delaminating crest [121, 157, 158]. Thus melanocytes, which in chick are the last to emigrate from the NT don't express *Foxd3* [123]. According to several reports, *Foxd3* is likely to be activated through distinct pathways from *Sox9* and *Snail2* [39, 117, 121]. *Sox9* deficient premigratory avian NC cells continue to express *FoxD3*. Furthermore overexpression of *Foxd3* gives a distinct phenotype from the ectopic expression of *Sox9* and *Snail2* [39]. *Xenopus* FoxD3 is slightly different as it works together with Zic in the early NC to induce *Snail2* [159]. Sox10 seems to be downstream of FoxD3 [122], although this may not be a direct connection given the 12 hour induction time shown in the avian overexpression experiments [39, 122] and one study could not see any change in *Sox10* or *Snail2* levels in the delaminating NC followed by a MO mediated *FoxD3* knockdown in zebrafish. The same study suggested that FoxD3 represses itself and *Snail1b* in the migratory crest [121].

The antiapoptotic Snail/Snail2 is needed for NC specification and cell cycle arrest: *Snail/Snail2* (*Snail* in mouse and fish, *Snail2* in chick and both in frog) is expressed in the developing dorsal neural folds and plays a role in the specification of the NC cells [24, 101, 104, 108, 160]. Ectopic overexpression of Snail2 increased the prospective neural crest area in the avian neural plate. However, in explant experiments Snail2 expression alone was not sufficient to induce NC without the presence of Wnt signaling (and to a lesser extent FGF-signaling [17, 39, 101]. In *Xenopus* the specification of NC in the neural plate is in fact induced Snail, which precedes the expression of *Snail2* [24, 161] and overexpression of Snail2 is also sufficient to induce NC [17]. *Snail* expression in *Xenopus* thus induces NC specifier genes such as *Snail2*, *Foxd3*, *Ets1* as well as *Twist*, which is a yet another known EMT inducer in cancer cells [24, 162] and ectopic Snail2 increases expression of avian *RhoB* and *Pax3* [55].

Another important role of *Snail2/Snail* in the presumptive NC is to promote cell survival by resisting Msx-mediated apoptosis. Snail2 downregulates the proapoptotic Caspase 9 and promotes activation of the antiapoptotic factor Bcl2. Loss of NC marker expression is

rescued by coexpression of BclXL after knockdown of Snail2, thus showing that Snail2 controls NC cell numbers [85, 160, 163]. Snail also induces cell cycle arrest by impairing the transition from early to late G1 by maintaining low levels of CyclinD and it can block the G1/S transition by maintaining high levels of p21. This suggests that *Snail* expression favors changes in cell shape versus cell division in order to enable EMT [85].

C-Myc and Id3 maintain self-renewal and induce proliferation: The widely studied basic helix-loop-helix transcription factor and oncogene c-Myc maintains self-renewal capacity in several embryonic and adult stem cells as well as cancer stem cells [164]. In the *Xenopus*, c-Myc controls NC specification at the neural plate border by maintaining multipotency and thus inhibiting premature fate determination, which is directly mediated through Id3 [165, 166]. Id3 also promotes NC proliferation associated with the survival of the NC progenitors at the neural plate border in the frog [167]. On the other hand, according to the avian expression pattern of the Myc-family members in the neural plate border, n-Myc might be responsible for the maintenance of multipotency in early NC since avian *c-Myc* is turned on only in the migratory NC [168]. c-Myc and n-Myc are known to function similarly during development [169] and they both regulate neural stem cell self-renewal in the mouse embryo [170–172]. The premigratory NC is highly proliferative as compared to the rest the NT and the cyclinD mediated transition (by phosphorylation based inactivation of Rb) of cell cycle regulation point from synthesis to mitosis (the S/G1 phase) has also been associated with the onset of NC emigration [78]. C-myc controls the S/G1 transition by direct activation of *cyclins D* and *E* transcription in many cell types [164]. Finally, the tumor suppressor p53 is expressed in the premigratory NC and coordinates growth, suggesting a role in self-renewal and NC specification and *p53* null mice also display severe craniofacial defects [173]. Even though more famous for its role ability to induce apoptosis and cellular senescence, p53 also controls self-renewal through repression of *c-Myc* in neural stem cells and glioma cancer stem cells [174, 175] as well as neural differentiation via cell cycle inhibition by activation of *p21* [176, 177]. Likewise, N-myc is associated with neural differentiation of NC derivatives in the ventrolateral pathway. Moderate levels of N-myc protein was detected in all the newly emigrated avian trunk NC cells but the expression was maintained only in the migratory derivatives that assumed a neural fate [178].

3.4. Nontranscriptional regulation of the EMT inducing transcription factors

3.4.1 Epigenetics—Silencing of genes by histone methylation and acetylation plays an important role in transcriptional regulation and phenotype maintenance. The histone demethylase Jumonji (JmjD2A) controls the timing of transcription of NC specifier genes. Occupation of avian JmjD2 on the *Sox10* and *Snail2* promoters, respectively, keeps them in an inactive H3K9 methylation state and JmjD2A is needed for the switch into the H3K36 methylated active chromatin, which thus allows transcription of the genes at the time of neural tube closure [179]. Similarly, *Pax3* expression pattern is also associated with active chromatin H3K4 and H3K36 methylation in mouse fibroblasts [180]. In ES cells Snail recruits components of the repressive Polycomb complex 2 as well as the SIN3A/histone deacetylase to the *E-cadherin* promoter [181, 182]. Foxd3, more known for its role as a repressor [183], is essential for providing an unmethylated mark at the Alb1 enhancer in ES cells. Overexpression of *Foxd3* in embryonic fibroblasts results in a loss of CpG methylation at the Alb1 enhancer and leads to induction of induced pluripotent stem (iPS) cells [184]. C-myc activity correlates with H3-K4 and H3-K79 active methylation marks as well as acetylation of Histone 3 [185]. C-Myc is known for its activation of the transcription of histone acetylases, demethylases and other chromatin modifying complexes as well as for their recruitment to the target promoters [164]. C-Myc induces maintenance of active chromatin in neural stem cells, mediated largely by the histone acetylase GCN5. Loss of n-Myc is associated with a loss of self-renewal capacity and condensed nuclei and terminal

differentiation of neurons [186]. Global chromatin condensation has also been associated with onset of migration by perhaps facilitating nuclear movement and reshaping [187].

3.4.2 Posttranslational modifications—The stability of the protein levels of the EMT regulators Snail, Snail2, Twist and Zeb2/Sip1 is regulated by a common mechanism in NC as well as in cancer cells mediated by the F-box protein Partner of paired (Ppa), which serves as the substrate recognition component of the Skp-Cullin-F-box E3 ubiquitin ligase. Ppa thus targets the proteins for degradation and misexpression of Ppa inhibits the formation of neural crest precursors [188–190].

SUMOylation, which interferes with the synergistic interactions of transcription factors to their binding co-factors, is a regulatory mechanism for conferring context-dependent function on widely deployed transcription factors such as the SoxE genes in NC. SUMOylation of *Xenopus* Sox9 and Sox10 is needed for their activity to induce ear development whereas non-SUMOylated Sox9 and Sox10 are active in NC progenitors and in the differentiating melanocytes [142, 191]. In the premigratory NC, *Sox10* is directly activated by Sox9, c-Myb and Ets1, whereas the onset of its expression in the otic placode is driven by a direct binding of a combination of paralogous transcription factors Sox8, c-Myb and Pea3. However, SUMOylation of Sox8 is required for the maintenance of *Sox10* expression in the otic placode [141, 192]. Sumoylation of other NC proteins such as Mx1, have also been reported but the meaning of that in the NC remains unknown [193].

3.4.3 miRNAs and alternative splicing—MicroRNAs have been identified as a new class of EMT regulators, in part owing to their regulation of EMT-inducing transcription factors [27, 194]. The miR-200 family of micro RNAs has an important role in EMT by directly targeting Zeb1/2 and vice versa thus leading to a feedback regulatory loop between EMT and MET [195]. Zeb mediated inhibition of a group of stemness inhibiting miRs (Mir200, Mir203 and miR-183), enhances the activity of self-renewal regulating genes *Bmi-1*, *Sox2* and *Klf4*, and thus combines stemness and EMT [196]. Stabilization and regulation of splicing by RNA binding proteins are yet additional RNA regulatory steps. Particularly ESRP1/2 have been shown to regulate a whole splicing program that promotes an epithelial phenotype and is thus repressed by EMT [27, 197]. These have yet to be examined in the neural crest system.

3.5 Sequence of events in NC EMT

Even though the series of events involved in EMT are well acknowledged, the sequence in which these steps take place has been under debate and it is not clear if EMT is a single event or can be achieved by multiple parallel mechanisms. The tissue of origin also sets up demands for the order of the EMT steps [198]. Migration studies have also elucidated an important point of the existence of intermediate migratory cells that have undergone EMT (e.g. downregulated *E-cadherin*) but still maintain a certain amount of polarity [27].

Several hypotheses, which need not be mutually exclusive, have been proposed to explain the onset of NC emigration from the NT. These include the loss of adherens junctions and apicobasal polarity [36, 38] or that the premigratory NC cells may generate a strong enough tractional force to pull themselves away from the adherens junctions without completely downregulating them [199, 200]. The timing of emigration is also regulated by the cell cycle as the G1/S transition has been reported to induce emigration [38, 49, 78, 201]. Interestingly, Snail blocks proliferation in the premigratory NC by arresting the cells in the G0/G1 -phase of the cell cycle. This may allow the cells to change shape as they are not actively dividing. It might also help synchronize cells so that they can all enter the S-phase upon being converted into mesenchymal cells for emigration to occur [85]. The advantage of

asymmetrically divided daughter cells that already are detached from the apical membrane was also thought to promote NC fate and emigration [199].

A 4D imaging study of chicken trunk slice cultures shows a surprisingly large variance of the order of events leading to the emigration of NC cells [202]. According to the study, during most of the documented EMTs, the cell was first detached from the apical surface followed by a retraction of the apical tail. After this the cell body translocated out of the epithelium thus suggesting that the downregulation of the apical adhesion complexes was the key step. However there were notable exceptions to this sequence since in around 30% of the cases the cell tail was ruptured while the apicobasal arrangement was still intact (as measured by visualization with α -catenin or actin, respectively) and tail fragments remained behind near the apical surface. Non-apical mitosis, which consisted of only 6% of all documented mitosis in the neuroepithelium, gave rise to 28% of the NC cells in total and in most of the cases both daughter cells became NC. These NC cells had already lost their apicobasal attachments and were rounded up in mitosis when they translocated towards the basal domain of the NT. These and other studies contradict the conclusion that the transition from G1 to S is the trigger of emigration and suggest that the rule may not apply to all NC cells [124, 202]. The cleavage plane of the cells attached to the apical membrane did not play a role in the decision of acquiring a NC fate, as asymmetric and symmetric dividing neuroepithelial cells displayed equal ability to become emigrating neural crest cells. The asymmetrically dividing daughter cells that had lost their contact to the apical membrane also are able to regain it [202]. Another recent live imaging study proposes that the fate of NC (whether to become glia, melanocytes, sensory neurons, peripheral nerves etc.) is already decided according to the location of the cell in the dorsal NT [203]. These two imaging studies also present controversial findings on the exit point of NC from the NT. According to Krispin and colleagues, NC cells only exit from the very dorsal tip of the NT whereas Ahlstrom and Ericson report emigrating cells in a more wide range up to a 90° angle [202, 203]. These studies, taken together, highlight the vast heterogeneity in the mechanisms that initiate NC emigration and raise many interesting questions about the mechanisms underlying neural crest EMT. The ultimate microenvironmental cues that influence these events remain to be shown. Clearly, more live imaging studies are needed to combine the knowledge of the fate-determining transcription factors with the morphology and behavior of the emigrating NC cells.

3.6 Mesenchymal to Epithelial transition

The arrest of neural crest migration is sometimes coupled with a reaggregation process, for example, during formation of peripheral ganglia and craniofacial cartilage. These cells undergo a mesenchymal to epithelial transition (MET). So far, surprisingly little is known about MET in the neural crest derived tissue. Whether the basis of regaining of the epithelial phenotype relies on reversal of the same molecular mechanisms occurring during EMT remains to be studied. Similarities e.g. include re-expression of N-cadherin in sympathetic ganglia, resulting in restoration of cell-cell adhesion and polarity [34, 204, 205]. The role of MET in the reversion of mesenchymal tumor cells, sometimes after long periods of dormancy, to a more epithelial state in distant metastases is a growing field of interest of cancer studies [194, 206, 207] and understanding how neural crest cells switch off their migration program and begin to differentiate may give important clues as to the prevention of tumor dissemination and spreading.

Highlights

- Neural crest (NC) cells undergo EMT to begin migration

- The neural crest (NC) is an excellent embryonic model for studying EMT under normal conditions
- NC EMT includes several steps from maintenance of multipotency to depolarization and emigration
- NC EMT may involve multiple parallel triggering mechanisms

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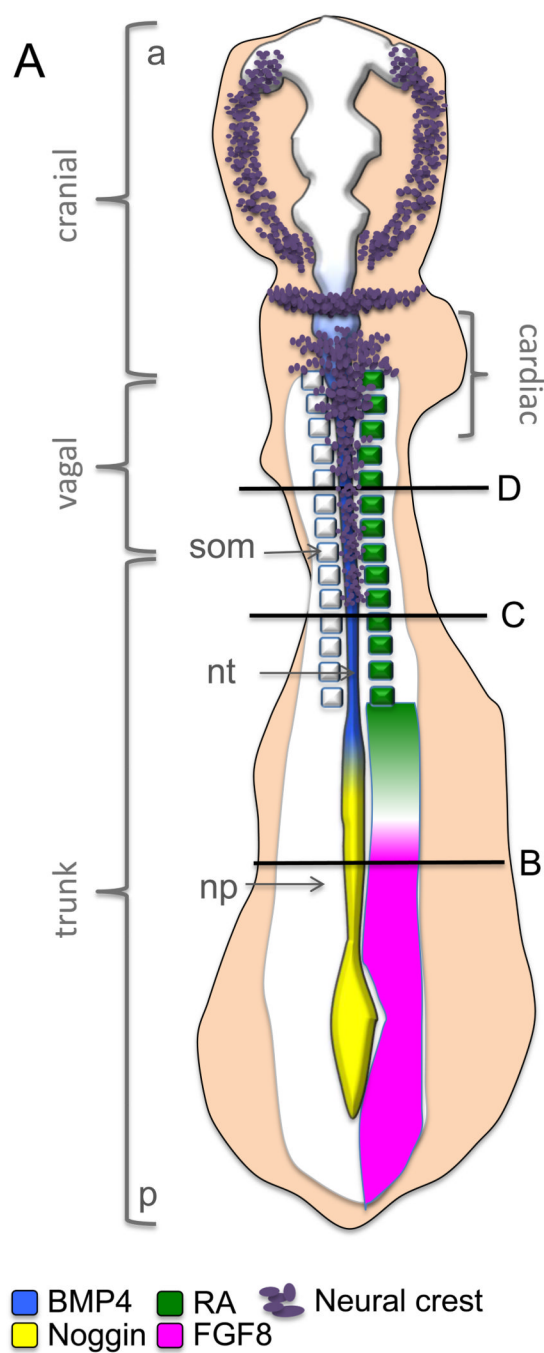
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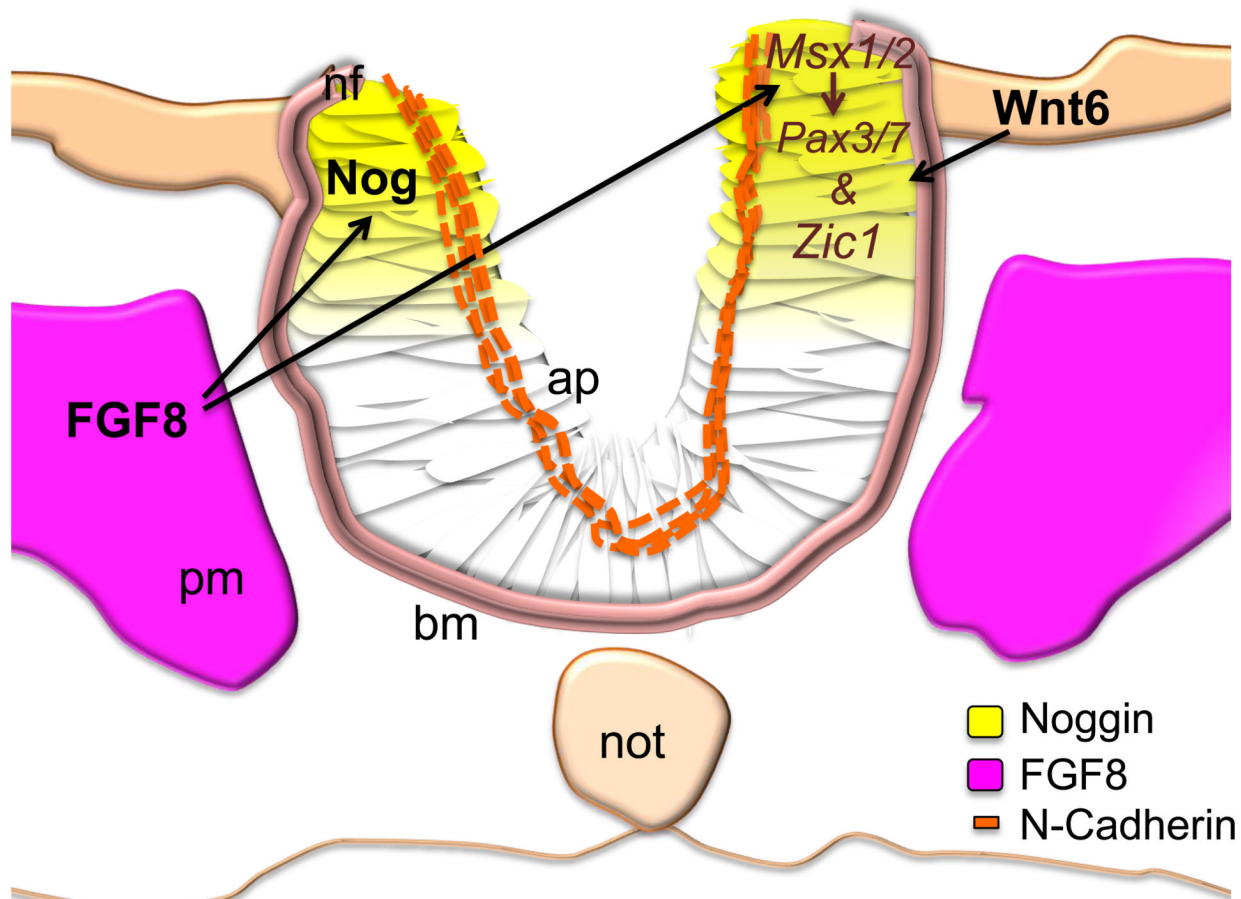
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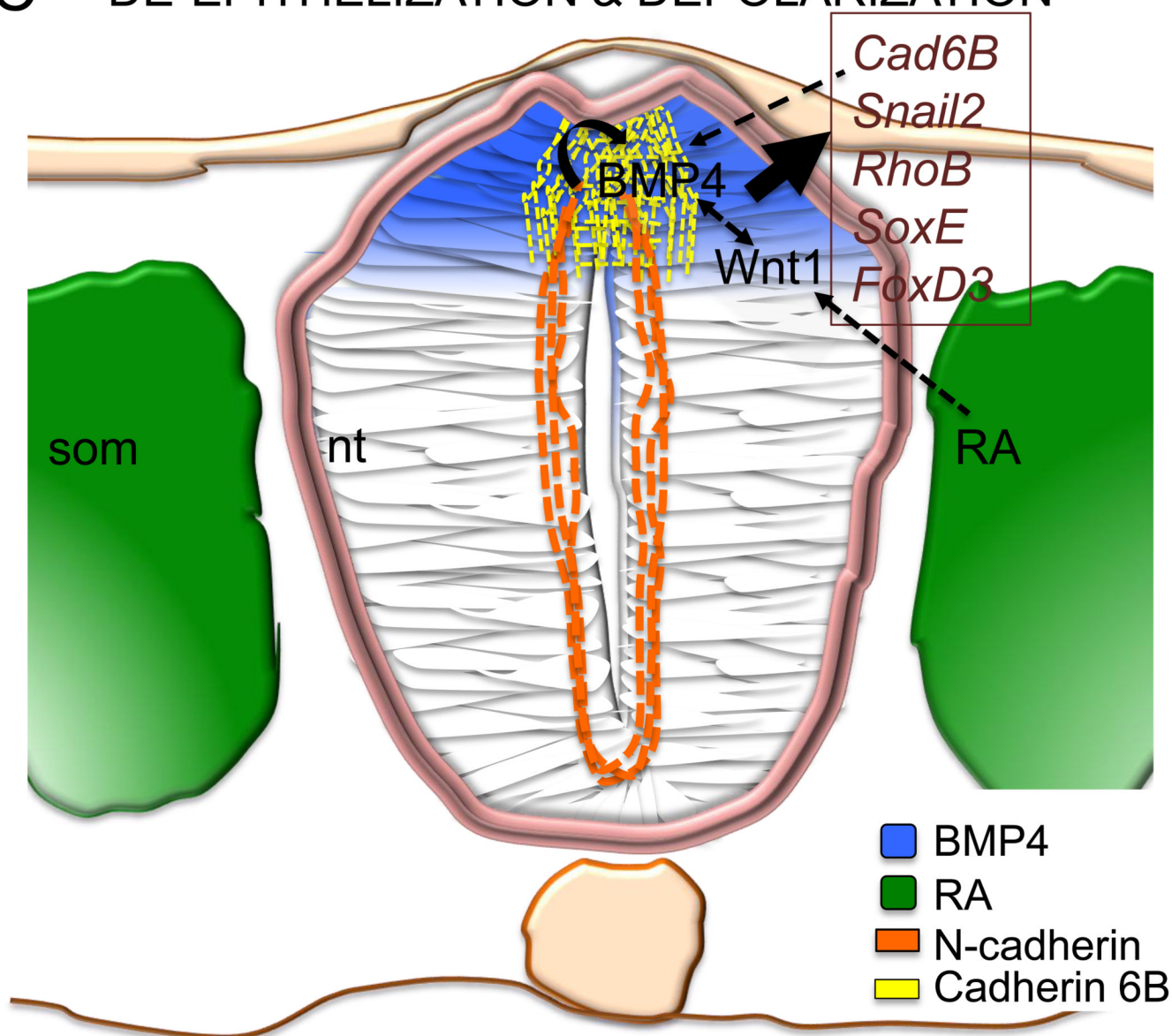
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B COMPETENCE & SPECIFICATION



C DE-EPITHELIALIZATION & DEPOLARIZATION



D

EMIGRATION & MIGRATION

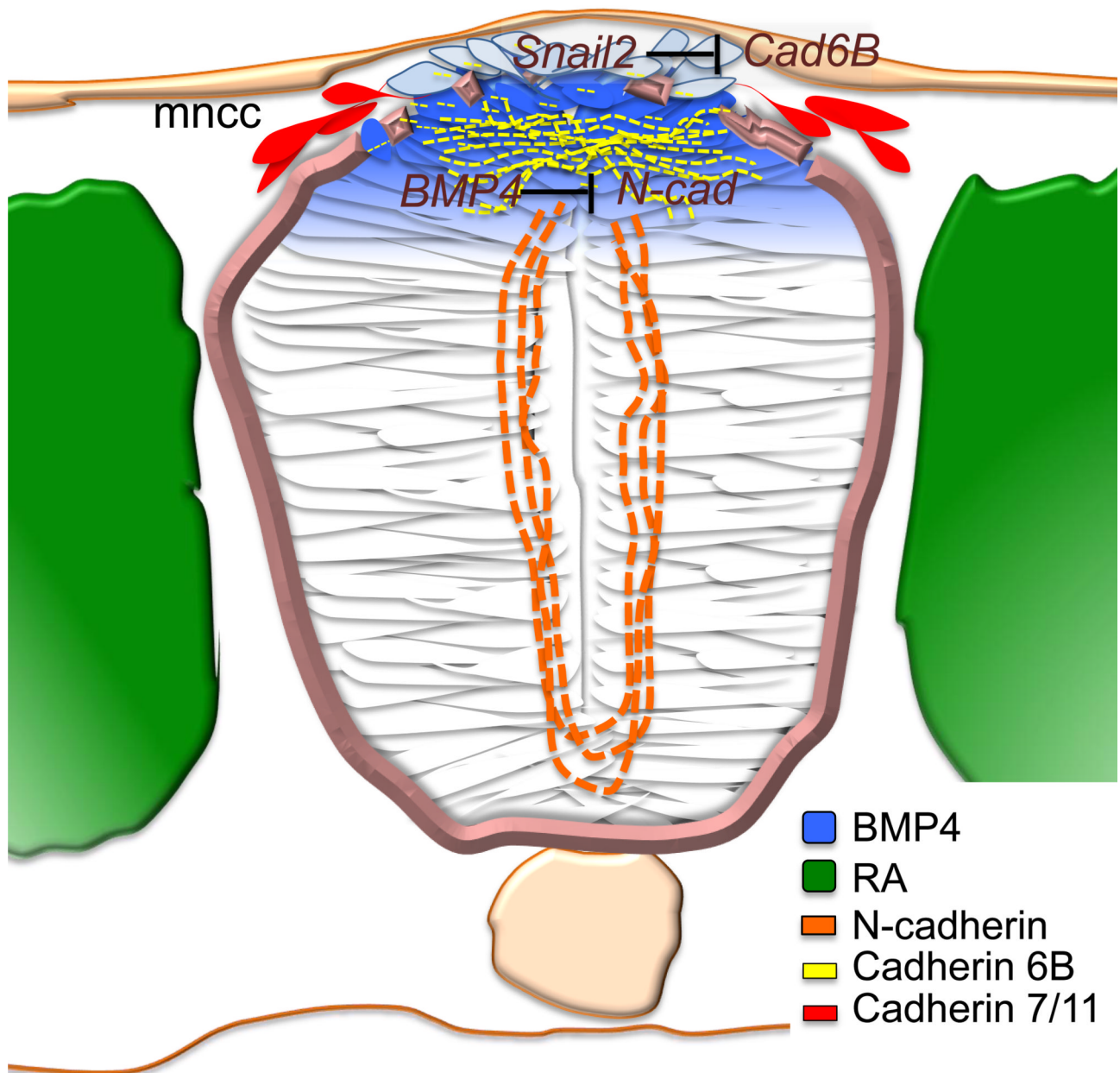
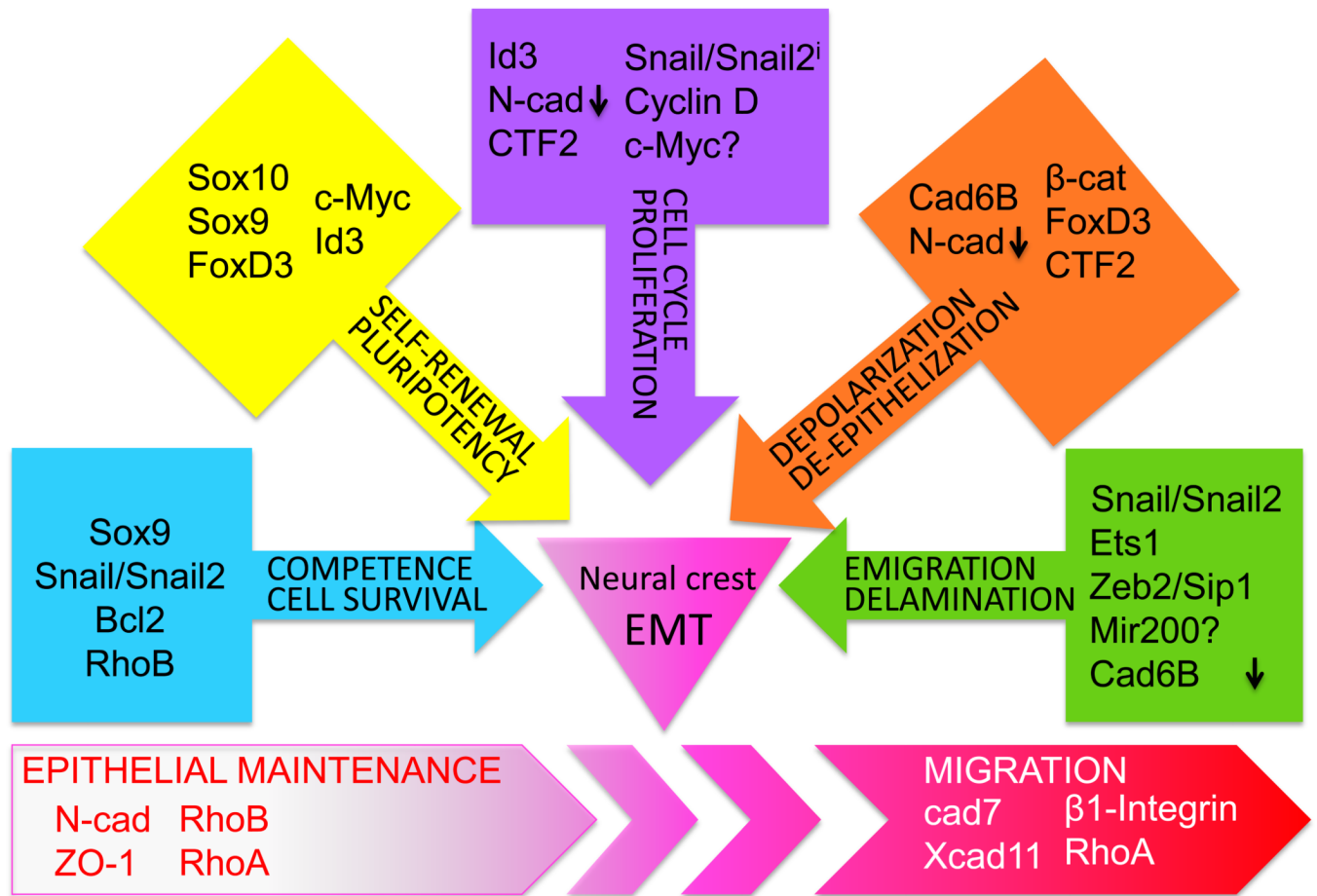


Figure 1.

Schematic of neural crest development in the chicken embryo showing that **(1A)** a rostrocaudal gradient of BMP4 is inhibited by caudally expressed Noggin. The BMP4 gradient in the neural tube, which mediates the timing of neural crest EMT, itself starts rostrally and proceeds caudally as the neural tube closes. FGF8 signaling from the paraxial mesoderm maintains Noggin and is inhibited by retinoic acid signaling (RA) also in a rostrocaudal gradient. Once EMT is complete, mesenchymal neural crest cells emigrate from the neural tube and migrate to their target sites. The black lines mark the axial levels at three transverse levels, representing the gradual progress of EMT (B–D). **(1B)** FGF secreted from

the paraxial mesoderm is required for the specification of the neural crest cells and induces expression of *Noggin* and *Msx1/2* in the dorsal neural folds. These in turn, in the presence of Wnt expressed by the non-neural ectoderm, drives expression of *Pax3/7* and *Zic1*. Apicolaterally bound adherens junction protein N-cadherin maintains the adhesion and polarity of the whole neural epithelium. **(1C)** RA secreted by the somites induces Wnt1, which induces BMPsignalling and inhibits Noggin. BMP4 is the inducer of the de-epithelization and depolarization factors in a subset of the dorsal neural tube cells that become neural crest. The apicolateral localization of N-cadherin is degraded and replaced by Cadherin 6B in the dorsal neural tube. **(1D)** The mesenchymal neural crest cells emigrate where the basal lamina is locally ruptured. *Cadherin 6B* is downregulated by *Snail2* and the migratory neural crest cells start to express *Cadherin 7/11*. som= somite, nt= neural tube, np= neural plate, a= anterior, p= posterior, nne= non-neural ectoderm, pm= paraxial mesoderm, bm= basement membrane, ap= apical side, not= notochord, nf= neural folds, mncc= migratory neural crest cell.

**Figure 2.**

The separate subdivisions of EMT that all need to be completed for the proper amount of multipotent neural crest cells to emigrate from the neural tube. This figure outlines the results of multiple studies obtained in several species. i=inhibitory role.